NMDA receptor-mediated calcium influx plays an essential role in myoblast fusion

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Abstract **Ca^{2+} influx is known to be prerequisite for myoblast fusion during skeletal muscle differentiation. Here, we show that the *N*-methyl-D-aspartate (NMDA) receptor is involved in the Ca^{2+} influx of C2C12 myoblasts. NMDA receptor (NR) 1 and NR2D were expressed in the myoblasts during muscle differentiation. Using Ca^{2+} imaging analysis, Ca^{2+} influx through NRs was directly measured at a single-cell level. L-Glutamate increased myoblast fusion as well as intracellular Ca^{2+} levels, and both effects were completely blocked by MK801, a selective antagonist of NRs. Furthermore, treatment with the Ca^{2+} ionophore A23187 recovered MK801-mediated inhibition of myoblast fusion. These results suggest that the NRs may play an important role in myoblast fusion by mediating Ca^{2+} influx.**

Keywords: *N*-Methyl-D-aspartate receptor; Ca^{2+} influx; Myoblast fusion; Muscle differentiation; C2C12 myoblast

1. Introduction

A prominent event in skeletal muscle differentiation is the fusion of mononucleated myoblasts into multinucleated myotubes and this myogenic process absolutely requires Ca^{2+} influx [1–3]. In addition, myoblast fusion is a fundamental process for muscle growth and repair in adults. We have previously shown that nitric oxide acts as a messenger molecule for myoblast fusion by mediating Ca^{2+}-dependent increase in intracellular cGMP level [4–6]. Unfortunately, the routes for the entry of external Ca^{2+} into myoblasts are still being debated.

Several different types of Ca^{2+} permeable channels have been proposed as mediators of Ca^{2+} influx in myoblasts. Bernheim and his colleagues have shown that T-type Ca^{2+} channels are expressed in postnatal human myoblasts and may play a crucial role in Ca^{2+} influx triggering myoblast fusion in a voltage-dependent manner [7,8]. Despite these findings, voltage-dependent calcium channels have not been detected in avian and rodent myoblast cultures [9,10]. In this context, several previous studies suggested alternative routes of Ca^{2+} influx for myoblast fusion, such as stretch-activated cation channels [10,11] or acetylcholine (ACh)-gated nicotinic channels [2,9]. These results give rise to two possibilities. One is that the Ca^{2+} conducting mechanism required for myoblast fusion is largely different according to the species and the other is that an increase in intracellular Ca^{2+} level large enough to trigger myoblast fusion requires the concerted effort of different types of channels which are permeable to Ca^{2+}.

It is well established that glutamate serves as a neurotransmitter at invertebrate neuromuscular junctions (NMJ) [12], whereas ACh serves this role in vertebrates. In addition, several studies in the last decade indicated a role for glutamate as a signaling molecule at the vertebrate NMJs [13]. In rat motoneuron terminals, glutamate-like immunoreactivity and high levels of mRNA for a glutamate transporter are present [14,15]. In addition, it was reported that *N*-acetylaspartylglutamate, its hydrolyzing enzyme *N*-acetylated α-linked acidic dipeptidase (NAALADase), and NR1 are found in rat diaphragm NMJs, using immunochemistry techniques [16]. These studies strongly suggest that *N*-methyl-*D*-aspartate (NMDA) receptors are involved in skeletal muscle NMJs.

The NMDA receptor (NR) belongs to a family of receptor-operated channels and is highly permeable to Ca^{2+} [17]. If this receptor is found in the early stages of muscle differentiation, it might function as a pathway for Ca^{2+} influx in myoblasts thereby triggering myoblast fusion. In this study, we sought to identify the expression of NR subtypes and determine whether Ca^{2+} influx was mediated by these channels in C2C12 myoblasts.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP was purchased from DuPont NEN. NMDA, MK801, and 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Research Biochemical Inc. Thapsigargin and calcium ionophore (A23187) were from Sigma Chemical. Cell culture reagents were obtained from Gibco Invitrogen.

2.2. Cell culture

C2C12 mouse skeletal muscle myoblasts were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in growth medium (Dulbecco's modified Eagle's medium, DMEM) containing 10% fetal bovine serum and 1% penicillin–streptomycin solution) at 37 °C under 9% CO_2. To induce differentiation from myoblasts to myotubes, the medium was replaced at 48 h after plating with a differentiation medium (DMEM containing 2% horse serum and 1% penicillin–streptomycin solution). When appropriate, drugs were added when the medium was changed.

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For the measurement of myoblast fusion, cells were fixed with 1% (v/v) glutaraldehyde for 30 min, stained with 0.1% hematoxylin for 30 min, and observed under a microscope at a magnification of 200×. Myoblasts were considered fused and counted only if there was clear cytoplasmic continuity and at least three nuclei were obvious. Each data point represents the mean of counts from more than 30 randomly chosen fields.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as described previously [18] with some modifications. Briefly, total RNA was extracted from C2C12 cells cultured for 8 days using Trizol reagent (Life Technologies). One microgram of the RNA templates was reverse-transcribed with SuperScript II RT (Life Technologies) using the manufacturer's instructions. Subsequently, each RT sample was subjected to PCR (a total of 30 cycles) using *Taq* polymerase (Perkin Elmer Cetus Corp., Norwalk, CT, USA). PCR primers for each NR subunit and non-NMDA (AMPA and KA) receptor subtype were chosen based on their sequence data [19–22] and were described in Table 1. After PCR amplification, the products were electrophoresed on 2% agarose gels and stained with ethidium bromide. All PCR products were cloned into pGEM-Teasy vectors (Promega) and sequenced.

2.4. Northern blot analysis

Total RNA was isolated from cultured C2C12 muscle cells at various time points using the Trizol reagent. The total RNA samples (30 μg each) were resolved on 1.2% formaldehyde agarose gels and transferred for 18 h by diffusion blotting to Nytran filters. ^{32}P-labeled antisense DNA probes to NR subunits were synthesized from linearized plasmids containing each PCR product using *Taq* polymerase in the presence of [α-^{32}P]dCTP and each 3′ primer. Hybridization procedures were performed as previously described [23]. After hybridization, membranes were exposed to X-ray film at −70 °C for 1–7 days. After autoradiography, the membranes were stripped and rehybridized with the control probes under the same conditions. Northern blot analyses were repeated at least three times.

2.5. Patch-clamp recordings

Electrophysiological recordings were done in cultured C2C12 cells using the whole-cell configurations of the patch-clamp technique. The bath solution contained (in mM): NaCl 165, KCl 2.5, $CaCl_2$ 0.5, glucose 10, HEPES 10, and glycine 0.001, pH adjusted to 7.4 with NaOH. The pipette solution contained (in mM): CsF 130, CsCl 15, CaCl2 0.5, EGTA 10, and HEPES 10, pH adjusted to 7.2 with CsOH. Holding potential was −40 mV. Currents were recorded at room temperature (20–23 °C) using an Axopatch 200A amplifier (Axon Instruments). Drugs were dissolved in external solution and applied to cells using a fast perfusion technique. A high-speed solution exchange system (HSSE-2, Ala Scientific Instruments Inc.) was used for the rapid application of glutamate. One mM glutamate was applied within 100 μm of the recorded cell allowing rapid application of drug.

2.6. Ca^{2+} imaging

Cells were cultured on gelatin-coated glass coverslips for 2 days and then loaded with 5 μM Fluo-3 AM (Molecular Probes, OR) by incubation for 60 min at room temperature in a normal bath solution [140 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, and 5.5 mM HEPES (pH 7.4)]. The Fluo-3 AM solution was removed by rinsing twice with the normal bath solution, then kept in it for 10 min at room temperature to complete ester-hydrolysis. To deplete stored intracellular Ca^{2+}, the cells were preincubated in the normal bath solution containing 1 μM thapsigargin for 30 min prior to experiments.

Intracellular Ca^{2+} concentrations were analyzed by the Fluo-3 AM fluorescence dye-detection method [24] using an inverted microscope equipped for epi-fluorescence and transmitted illumination (IX-70; Olympus America, NY, USA). Fluorescent images were obtained using a 40× objective lens; the excitation light source was a xenon lamp coupled to excitation filter wheels. The emission filter wheel was coupled to the microscope at the camera output port. Images were captured using a slow scan, liquid nitrogen-cooled, charge-coupled device (CCD) camera with a back-thinned, back-illuminated imaging chip (Quantix, Photometrics, AZ, USA). Optical excitation for Fluo-3 was accomplished with a 488 nm wavelength of xenon light. The emitted fluorescence was passed through a 535 nm emission filter before it reached the CCD camera. Fluorescent images were captured in a slow mode (1 frame/5 s). An average brightness over time plot was constructed for a defined intracellular area with the plots corrected for dye bleaching using an exponential decay algorithm (MetaFluor, Universal Imaging, PA, USA). The data were presented as the average of normalized fluorescence ratio (F/F_0) of 10 individual cells. All experiments were repeated at least three times.

Table 1
Primers used for RT-PCR

Gene	Primer sequence	Size (bp)
NR1	F: 5′-AGTCCCTTTGGCCGATTTAA-3′ R: 5′-ACCAGATCGCACTTCTGTGA-3′	459
NR2A	F: 5′-TGCA-AACATTTGTGGCCAAC-3′ R: 5′-TTGGTCTTGTGGTTCTTATT-3′	361
NR2B	F: 5′-AGCATCGGCATCGCTGTCAT-3′ R: 5′-GCCAAACTGGAAGAACATGG-3′	342
NR2C	F: 5′-GGAGACACAG-AAGTTGGAGA-3′ R: 5′-ACGTCCTCTGTGCTCACCAT-3′	375
NR2D	F: 5′-CGCCTCAAGTACCCTCTATG-3′ R: 5′-GACGCCATCG-ATCTTCTTGC-3′	333
AMPA	F: 5′-CCTTTGGCCTATGAGATCTGGATGTG-3′ R: 5′-TCGTACCACCATTTGTTTTTCA-3′	749
KA	F: 5′-TGGGCCTTCACCTTGATCATCA-3′ R: 5′-CTGTGGTCCTCCTCCTTGGG-3′	512

2.7. Statistical analysis

Statistical comparisons were analyzed by the unpaired Student's *t* test. A probability level of $P < 0.05$ was selected for determining statistically significant differences.

3. Results

3.1. Presence of NR subtypes in C2C12 myogenic cells

It has been known that there are three distinct ionotropic glutamate receptor subtypes in neuronal cells: NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA), and the kainite (KA) receptor subtype [17]. To determine which types of glutamate receptors are expressed in C2C12 myoblasts, RT-PCR was performed using glutamate receptor subtype-specific PCR primer sets (Table 1).

As shown in Fig. 1, both NMDA and non-NMDA (AMPA and KA) receptors were expressed in C2C12 cells, although all subunits of NR were not found. NRs consist of NR1 and NR2A-2D subunits; the NR1 subunits are in hetero-oligomeric configurations with NR2A-2D [25]. NR1 and NR2D were predominantly expressed, while NR2A was only slightly expressed in C2C12 cells, whereas all types of NR2 subunits were detected in the mouse whole brain, used as a positive control (Fig. 1A). These PCR products were sequenced and the results showed complete consensus with those previously reported [19–22]. We then determined the expression pattern of NR subunits during muscle differentiation using Northern blots. The expression of the NR1 and NR2D subtypes gradually increased during differentiation (Fig. 1B); the NR2A subtype was not detected (data not shown).

3.2. Effect of glutamate and NMDA on intracellular Ca^{2+} levels in myoblasts

It is reasonable to assume that these NR subtypes are functional and may be responsible for the Ca^{2+} influx necessary for myoblast fusion. To examine this possibility, we carried out

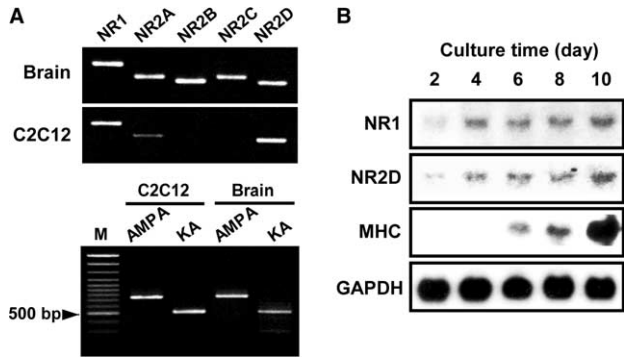

Fig. 1. Expression of ionotropic glutamate receptor subtypes in mouse C2C12 cells. (A) RT-PCR product of NMDA subunits (upper and middle panel) and non-NMDA subtypes (lower panel) from mouse brain and C2C12 myoblasts. PCR was performed using specific primers for NR subunits (NR1 and NR2A-2D) and non-NRs (AMPA and KA). (B) Expression of NR subunits during skeletal muscle differentiation. Total RNAs obtained from the cells cultured for the indicated periods and subjected to Northern blots analysis using NR1, NR2D, myosin heavy chain (MHC), and GAPDH probes. MHC and GAPDH were shown as an indicator for myogenic differentiation and the amount of the RNAs, respectively. Similar data were obtained in three independent experiments.

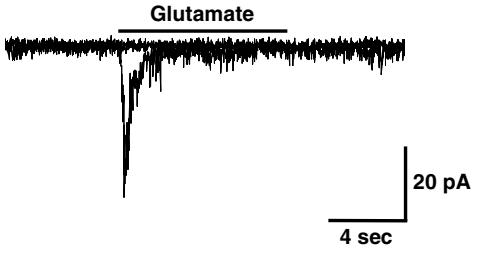

Fig. 2. Whole-cell recording of glutamate channels in C2C12. Application of 1 mM glutamate produces an inward current response at −40 mV with transient components. For the rapid application, drug was applied within 100 μm of the recorded cell using a high-speed solution exchange system.

electrical recordings and Ca^{2+} imaging experiments in C2C12 myoblasts. Treatment with L-glutamate induced an inward current when the membrane potential was clamped at −40 mV (Fig. 2) and increased intracellular Ca^{2+} level in a dose-related manner and this effect was maximal at 10 mM (Fig. 3A and B). In addition, treatment with NMDA, a non-metabolic agonist of NRs, showed similar increases in intracellular Ca^{2+} levels to glutamate at the same concentration (Fig. 3A and C). These effects might be due to external Ca^{2+}, as pretreatment with EGTA (2 mM) completely blocked the increase in intracellular Ca^{2+} levels by glutamate (data not shown).

To further clarify the involvement of NRs in Ca^{2+} influx of C2C12 myoblasts, we depleted myoblast intracellular Ca^{2+} stores by preincubation with thapsigargin (1 μM), an inhibitor of microsomal Ca^{2+}-ATPase [26], for 30 min (Fig. 4A), and examined the glutamate-induced increase in intracellular Ca^{2+} levels with either antagonists of glutamate receptors or not. Depletion of intracellular Ca^{2+} stores did not block the increase in intracellular Ca^{2+} levels by L-glutamate (Fig. 4B); this effect was completely inhibited by 0.1 mM MK801, a selective antagonist of NR (Fig. 4C). In contrast, 0.1 mM CNQX, a non-NR inhibitor, did not affect the glutamate-induced Ca^{2+} influx (Fig. 4D). These results strongly suggest that Ca^{2+} influx could occur through NRs in C2C12 myoblasts.

3.3. Role of NRs on myoblast fusion

Ca^{2+} influx is known to be essential for myoblast fusion [3]. In cultured myoblasts, intracellular Ca^{2+} levels were effectively increased by treatment with glutamate. Thus, we investigated the role of NRs in myoblast fusion. C2C12 cells were treated with increasing amounts of L-glutamate 2 days after plating and assessed for the cell fusion 6 days later. As shown in Fig. 5, glutamate promoted myoblast fusion dose-dependently, as did NMDA (up to 5 mM) (data not shown), while MK801 dramatically inhibited spontaneous myoblast fusion as well as the glutamate-induced myoblast fusion. Both effects were dose-dependent, and the effect of glutamate was maximal at 10 mM, the same concentration producing maximal intracellular Ca^{2+} levels (Fig. 3). Upon treatment of myoblasts with MK801 at all concentrations tested, there was no significant effect on cell proliferation as assessed by [^{3}H]thymidine incorporation (data not shown). Moreover, MK801-mediated inhibition of spontaneous myoblast fusion was significantly recovered by treatment with Ca^{2+} ionophore A23187 (Fig. 5C). Thus, the effect by MK801 appears to be not due to simple cytotoxicity but caused by inhibiting Ca^{2+} influx through NRs.

4. Discussion

There is considerable evidence that NRs mediate a fast excitatory synapse transmission in the vertebrate central nervous system (CNS) and are localized in both presynaptic and postsynaptic neurons [17]. Although prior studies on this channel were usually focused on the CNS, some recent evidence shows that functional NRs are not restricted to neurons but are also expressed in several non-neuronal cells such as human keratinocytes [27], rat pancreas [28,29], and cultured myocardiocytes [27,28]. In the rat and mouse skeletal muscles, the NR subunit R1 was found in NMJs [16,30]. It was also reported that NR1 and the ACh receptor (AChR) were linked to nitric oxide synthase 1 in C2C12 skeletal myotubes [31]. These results imply that functional NRs may exist in skeletal myotubes.

While NR1 was found in mature muscle [16,30,31], the expression of NRs in myoblasts has not yet been determined. The present study provides the first evidence for NR subtype expression in myoblasts and their involvement in muscle differentiation. C2C12 skeletal myotubes do express NR subtypes such as NR1, NR2A, and NR2D. Also, glutamate and NMDA increase Ca^{2+} levels in C2C12 myoblasts, implying that NRs are one pathway for Ca^{2+} influx, which is a prerequisite for myoblast fusion.

It is of importance to determine whether motoneurons are able to release glutamate as a neurotransmitter at NMJ. *N*-Acetylaspartylglutamate, a dipeptide localized in putative glutamatergic neurons in the brain, is found in mammalian motoneurons [32]. This dipeptide is a partial agonist of NRs in the brain [33] and can be cleaved into glutamate and *N*-acetylasparate by the N-acetylated α-linked acidic dipeptidase (NAALADase) [34]. Since this dipeptide is also detected at the NMJ [30], it is possible that glutamate might be released by motoneurons and is thus involved in muscle differentiation.

In the present study, we clearly show that the NR is a strong candidate as a channel responsible for Ca^{2+} influx in

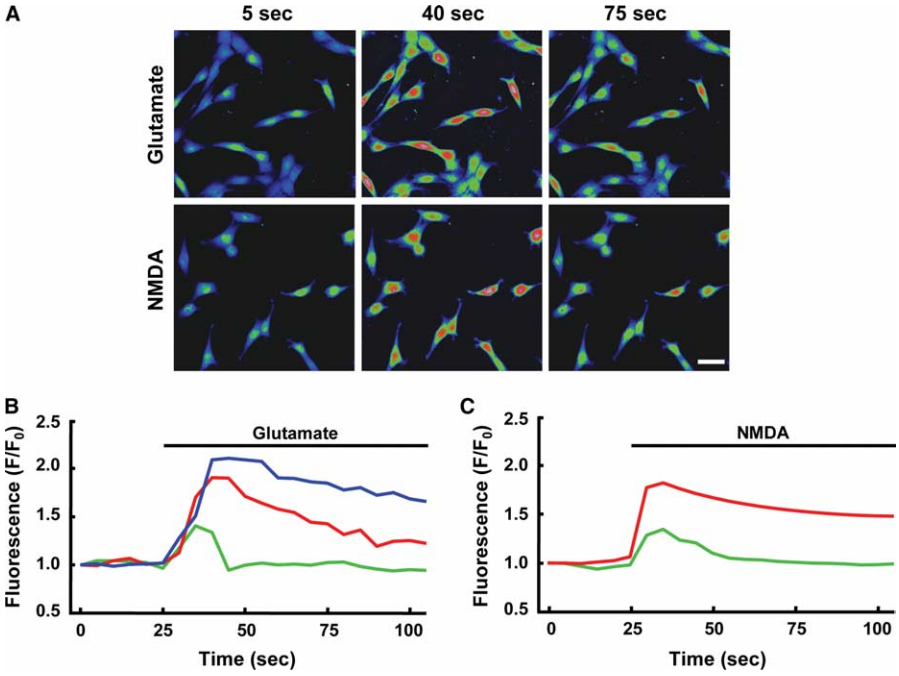

Fig. 3. Effect of glutamate and NMDA on intracellular Ca^{2+} levels in C2C12 myoblasts. Intracellular Ca^{2+} levels were monitored by Fluo-3 fluorescence. The vertical axis represents relative fluorescence intensity normalized to that of the basal level (F/F_0). (A) Fluorescence images of Fluo-3 loaded cells during typical experiments. Images were presented at the indicated time, before (5 s) and after (40 s, 75 s) stimulation with glutamate or NMDA (5 mM). Scale bar, 50 μm. (B) Changes in intracellular Ca^{2+} levels were monitored in the absence and presence of increasing amounts of glutamate: 1 mM, green line; 5 mM, red line; 10 mM, blue line. (C) Changes in intracellular Ca^{2+} levels were monitored in the absence and presence of increasing amounts of NMDA: 1 mM, green line; 5 mM, red line. All Ca^{2+} imaging analyses were repeated at least three times.

myoblasts, thus playing a significant role in triggering myoblast fusion. The overall mechanism of Ca^{2+} mobilization in myoblasts is, however, still a matter of debate since various types of Ca^{2+}-permeable channels including T-type Ca^{2+} channels, nicotinic AChRs and stretch-activated channels have been suggested as pathways responsible for the Ca^{2+} influx in myoblasts [7–11]. Recently, it was reported that myoblast fusion is not controlled by an all-or-none type Ca^{2+} signal

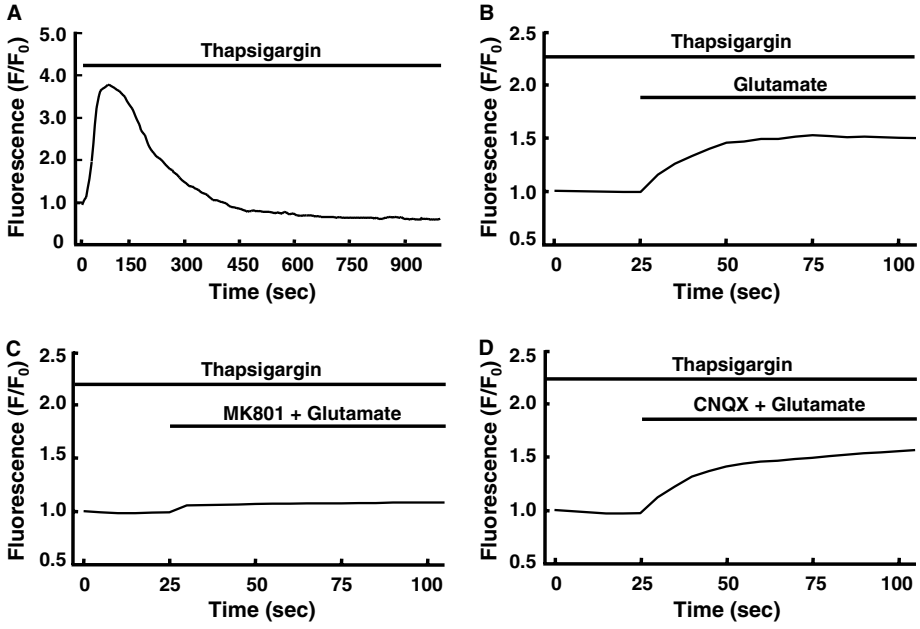

Fig. 4. Involvement of NRs in myoblastic Ca^{2+} influx. Changes in intracellular Ca^{2+} levels were monitored by normalized Fluo-3 fluorescence ratio (F/F_0). Pretreatment with thapsigargin (1 μM) for 30 min elicited complete depletion of intracellular Ca^{2+} stores in C2C12 cells (A). After intracellular Ca^{2+} depletion, changes in intracellular Ca^{2+} levels were monitored by treating the cells with 10 mM glutamate in the absence (B) and presence of 0.1 mM MK801 (C) or 0.1 mM CNQX (D).

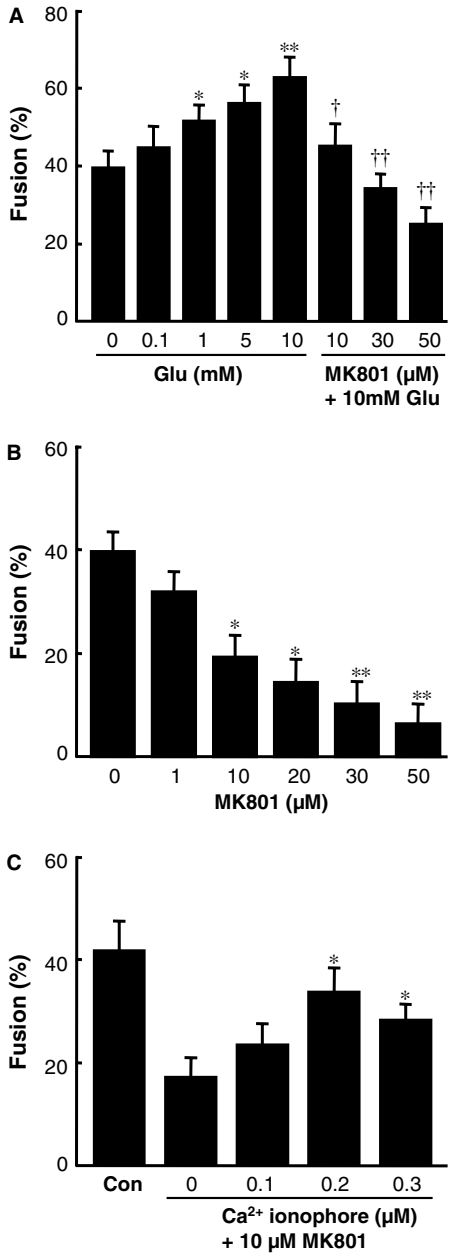

Fig. 5. Effects of glutamate and MK801 on myoblast fusion. C2C12 myoblasts were treated with the indicated amounts of the drugs 2 days after plating; the extent of fusion was assessed at 8 days. Each bar represents the mean SE of four independent cultures. (A) Effect of glutamate on myoblast fusion in the presence and absence of MK801. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs. 10 mM glutamate-treated group. (B) Dose-dependent effect of MK801 on spontaneous myoblast fusion. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated group. (C) Effect of Ca^{2+} ionophore on reversal of MK801-mediated inhibition of myoblast fusion. Cells cultured for 2 days were incubated for 2 days in the presence or absence of 10 μM MK801. After the incubation, they were treated with increasing amounts of Ca^{2+} ionophore (A23187) and cultured for 4 more days. $^{*}P < 0.05$ vs. 10 μM MK801-treated group.

but by a graded one and, therefore, any mechanism able to modulate intracellular Ca^{2+} concentration could affect the rate of myoblast fusion [8]. All these findings increase the possibility that myoblast fusion might be triggered by the combined effect of Ca^{2+} influxes through different types of Ca^{2+} permeable channels. In this regard, our present results are compatible with previous findings and are suggestive of the fact that Ca^{2+} influx through NRs contribute, at least in part, to increasing the intracellular Ca^{2+} concentration required to trigger myoblast fusion.

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